Immune-mediated mechanisms influencing the efficacy of anti-cancer therapies

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Summary

Conventional anti-cancer therapies, such as chemotherapy, radiotherapy and targeted therapy, are designed to kill cancer cells. Yet, the efficacy of anti-cancer therapies is not only determined by their direct effects on cancer cells, but also by off-target effects within the host immune system. Cytotoxic treatment regimens elicit a number of changes in immune-related parameters including the composition, phenotype and function of immune cells. In this review, we discuss the impact of innate and adaptive immune cells on the success of anti-cancer therapy, we examine the opportunities to exploit host immune responses to boost tumor clearing and we highlight the challenges facing the treatment of advanced metastatic disease.

Then and now: The link between the immune system and anti-cancer therapies

The relationship between anti-cancer therapies and the immune system is as old as the invention of anti-cancer therapies themselves. After the use of mustard gas in the trenches of World War I, a seminal observation was made that some exposed soldiers displayed severe loss of bone marrow and lymph node cells [1]. This observation then spurred the idea that the anti-proliferative capacity of mustard gas may also slow the growth of cancer cells. Experiments carried out in mice transplanted with lymphoid tumors were convincing enough to treat a lymphoma patient [2], and these events initiated the standardized treatment of cancer patients with chemotherapy [3, 4].

Fast forward 100 years later. The influence of immune cells on tumor progression and metastasis is well established [5], and an appreciation for the immune system's impact during conventional anti-cancer therapy treatment is growing. Recent seminal advances indicate that immune cells can shape the outcome of various anti-cancer therapies. As such,

immune cells and their molecular mediators have evolved into *bona vide* targets of therapeutic manipulation in cancer patients. The recent breakthrough of immunotherapeutics that inhibit negative immune regulatory pathways, such as anti-CTLA4 and anti-PD1, has initiated a new era in the treatment of cancer [6]. In parallel, immunomodulatory strategies aimed at dampening pro-tumor functions of immune cells are currently being tested in cancer patients [7]. Immune cells also function as reliable biomarkers, since their abundance or activation status often predicts how well patients respond to a particular treatment regimen. Here, we review these novel experimental and clinical insights, highlighting potential implications for the development of synergistic therapies designed to combat primary tumors and, more importantly, metastatic disease.

The pros and cons of experimental mouse models

Research questions aimed at understanding the role of immune cells during anti-cancer therapy require models that mirror the complex interactions between the immune system and diverse forms of human cancers. Transplantable cancer cell line models and carcinogen-induced cancer models are the most frequently used for these purposes. However, studies in genetically engineered mouse models (GEMMs; see Glossary) that spontaneously develop specific cancer types as a consequence of germline or somatic mutations in discrete cell types are gaining ground. There are key differences between cancer cell line inoculation models and GEMMs of cancer (**Box 1**). In GEMMs, normal cells are transformed *in situ* resulting in the development of spontaneous tumors that faithfully recapitulate each stage of cancer progression – from tumor initiation to advanced disease and in some models also metastasis. These spontaneous tumors develop in their natural microenvironment, and share the genetic heterogeneity and histopathology of human tumors. In stark contrast, transplantable models rely on the inoculation of large numbers of selected, homogenous cancer cells grown in 2D. The tissue of tumor origin and location of injection are often disparate in transplantable models with subcutaneous injection being the most common site of implantation. Moreover, these tumor cell line inoculation models do not mimic the multi-step progression of *de novo* tumors, and the speed of tumor outgrowth is very fast. Upon inoculation, a large proportion of the cancer cells die, which can prime anti-tumor immune responses in an unphysiological manner. Importantly, comparative studies have shown that immune cell behavior and tumor response to anti-cancer therapies differs between transplantable cell lines derived from GEMMs and the original GEMM [8-10]. Similarly, other studies indicate that the use of GEMMs in pre-clinical studies may be better predictors of clincial trials than transplantable models [11]. Xenografted human cancer cells established from cell lines or fresh patient material (patient-derived xenograft, PDX) in immunocompromised mice are other frequently used models. While it may be argued that

PDX models are the best representation of human disease from a cancer genetics or drug response point-of-view, these models exclude the participation of the adaptive immune system in cancer progression and anti-cancer therapy response. Therefore, they cannot predict the full breadth of drug response in immunocompetent humans. These issues, as well as other advantages and disadvantages, various strategies to refine these models and their suitability for pre-clinical studies, have been extensively discussed elsewhere [12-16].

The influence of the immune system on chemotherapeutic efficacy

Various types of chemotherapy drugs exist which kill cancer cells via different mechanisms (**Figure 1**). Cytotoxic drugs can eliminate cancer cells by inhibition of DNA replication, chemical damaging of DNA, inhibition of the function of critical enzymes required for DNA synthesis or prevention of mitosis. Drug-induced cancer cell death, as well as off-target effects of chemotherapy, elicits a number of systemic and intra-tumoral changes in the host immune system. In turn, the efficacy of chemotherapeutic drugs is influenced by the interplay between tumor and immune components. These mechanisms are outlined below for both innate and adaptive immune cells.

Innate immune cells

The microenvironment of solid tumors consists of multiple cell types, including many immune cell populations that participate in and regulate tumorigenesis and metastasis [17, 18] (**Box 2**). Tumor-associated macrophages (TAMs) represent one of the most extensively studied innate immune cell populations in chemotherapy response. Research spanning over the last three decades has shown that TAMs interfere with or augment the therapeutic activity of several types of chemotherapy and their role in these processes has been reviewed recently [19, 20]. One of the first studies addressing the impact of macrophages on chemo-responsiveness showed that doxorubicin enhances the tumoricidal properties of TAMs in mice transplanted with leukemia and lymphomas and that macrophage-inactivating agents reduce the efficacy of doxorubicin. Interestingly, these observations were chemotherapy specific, since daunorubicin, another anthracycline, together with TAM depletion failed to exhibit any synergism [21].

More recent literature pertaining to TAMs and solid epithelial malignancies indicates a sinister role for these cells in limiting chemotherapy efficacy. Increased TAM abundance and low $CD8^+$ T cell abundance in human breast tumors is associated with poor response to neoadjuvant chemotherapy [22]. Paclitaxel treatment of mammary tumor-bearing *MMTV-PyMT* mice increases TAM infiltration into tumors. These cells counteract chemotherapy efficacy via several mechanisms, including inhibition of anti-tumor $CD8^+$ T cell responses via IL10-mediated suppression of dendritic cells [22, 23], as well as secretion of chemoprotective

survival signals, such as cathepsins [24]. Interestingly, splenic macrophages have also been implicated in conferring systemic resistance to cisplatin in subcutaneous cell line models via secretion of lysophospholipids that alter DNA damage response [25].

In this regard, various strategies to deplete TAMs or neutralize their mediators have been used to enhance chemotherapy efficacy in preclinical tumor models. The most common strategy used to date involves the inhibition of CSF1-CSF1R signaling, since these molecules are required for macrophage differentiation and maturation [26, 27]. Paclitaxel treatment in combination with a CSF1R inhibitor reduces tumor growth in both *MMTV-PyMT* and *C3(1)-Tag* mice [22, 23] – a conditional GEMM driven by SV40 large T antigen in mammary epithelial cells [28]. Of note, this therapy combination also decreases spontaneous lung metastasis in *MMTV-PyMT* mice, while neither paclitaxel nor CSF1R inhibitor alone affects metastasis formation [22, 23]. Similarly, when paclitaxel is used with an inhibitor blocking TAM-derived cathepsins, the total lung metastatic burden of *MMTV-PyMT* mice is lowered [24]. In orthotopic pancreatic tumor transplants, blockade of TAMs and monocytes via CSF1R or CCR2 inhibitors synergizes with gemcitabine and paclitaxel to slow cancer growth and to reduce peritoneal metastasis [29]. Like TAMs in *MMTV-PyMT* mammary tumors [22], TAMs from these pancreatic tumor transplants suppress $CD8^+$ T cell activation to foster chemoresistance [29]. Studies in xenograft tumor models using human breast cancer cell lines have also shown that CSF1 neutralization together with a triple chemotherapy modality (cyclophosphamide, methotrexate, and 5-FU) reverses chemoresistance [30]. Another chemotherapeutic drug, trabectedin, induces apoptosis specifically in monocytes and macrophages, and this forms a key component of its anti-tumor activity [31].

Although these studies reveal that macrophages counteract the efficacy of various chemotherapeutics and suggest that the synergism between TAM inhibition and chemotherapy may be beneficial for several types of cancer, it will be important for future experiments to focus on resistance mechanisms within the immune system. The studies above combining chemotherapy with TAM blockade show only a transient effect on tumor growth; these tumors do not regress and they eventually grow out [22, 23, 29, 30]. The inherent flexibility and redundancy of the immune system lends itself to potential deleterious feedback mechanisms in which the functions of a depleted population are reinstated by another population. One such mechanism is known to occur in cervical tumors of *K14-HPV/E$_2$* mice, where genetic inhibition of MMP9-expressing TAMs results in a compensatory neutrophil influx that restores MMP9 levels, angiogenesis and tumor progression [32]. Similarly, inhibition of TAMs via CSF1R in mammary tumor-transplanted mice induces a neutrophil-dependent increase in lung metastasis without affecting primary tumor growth [33].

The resistance mechanisms counteracting the synergism between chemotherapy (or other anti-cancer therapies) and TAM blockade remains to be seen.

The CSF1 and CSF1R inhibitors utilized in the reports above function by depleting the entire TAM population [22-24, 29, 30]. However, another promising strategy that may prevent resistance to TAM depletion is to switch the polarization of TAMs from a pro-tumorigenic phenotype to a more anti-tumorigenic phenotype (**Box 3**). This concept was recently demonstrated in a GEMM of glioblastoma [34] and an orthotopic pancreatic cancer model [35], where CSF1R inhibitors reduce expression of immunosuppressive and pro-angiogenic genes and increase immunostimulatory genes in TAMs. Overexpression of a molecule called histidine-rich glycoprotein (HRG) also repolarizes TAMs [36]. Resident TAMs in transplanted fibrosarcomas overexpressing HRG exhibit a more anti-tumorigenic, less angiogenic phenotype than TAMs in control tumors. This phenotypic switching renders tumors more sensitive to doxorubicin by modulation of the tumor vasculature [36]. There is also evidence that type I interferons convert TAMs into tumor-antagonizing cells. TAMs engineered to express IFNα upregulate expression of dendritic cell markers in mammary tumors of *MMTV-PyMT* mice. As a consequence, these mice exhibit increased tumor-infiltrating effector $CD8^+$ T cell frequencies, slower tumor growth and lower incidence of metastasis [37]. As such, the combination of chemotherapy together with drugs that repolarize TAMs may be exploited to achieve greater patient responses and prevent resistance mechanisms within the immune system. Some chemotherapeutics skew the polarization of macrophages directly [38], or indirectly via regulating cancer cell-secreted factors [39].

Intravital imaging of experimental tumor models has provided additional clues about the behavior of TAMs after chemotherapy. In these studies, doxorubicin treatment induces infiltration of CCR2-expressing monocytes into necrotic regions of *MMTV-PyMT* mammary tumors, where these cells control vascular permeability and facilitate regrowth of tumors through MMP9 expression [40]. Consequently, spontaneous and transplanted tumors grown in *Ccr2* or *Mmp9* knockout mice acquired an increased sensitivity to doxorubicin [40]. These studies suggest that TAMs control drug delivery through regulating vessel functionality and leakiness. In support of this notion, deletion of pro-angiogenic molecules, such as VEGFA and PlGF, in myeloid cells and bone marrow-derived cells, respectively, decreases vascular leakiness [36, 41] and increases the potency of cyclophosphamide on transplanted tumors [41].

Like TAMs, the role of tumor-associated neutrophils in response to chemotherapy treatment is context and tumor type dependent. In athymic nude mice bearing E1A/Kras/Bcl-xL-transformed murine embryonic fibroblast (MEF) tumors, the depletion of neutrophils using the Gr1 antibody impairs the anti-cancer effect of cyclophosphamide [42]. The Gr1 antibody

binds the granulocyte-specific antigen, Ly6G, as well as the Ly6C antigen that is expressed by both granulocytes and monocytes. So it is possible that $Ly6C^+$ inflammatory monocytes may also contribute to tumor control in this scenario. Neutrophil depletion via the more specific anti-Ly6G antibody also modestly impairs the anti-cancer effect of doxorubicin on various cancer cell lines transplanted into syngeneic, immunocompetent mice [43].

By contrast, strategies to impede neutrophil recruitment into tumors augment the efficacy of chemotherapy. This has mainly been accomplished by inhibiting CXCR2, the receptor for CXCL1, 2 and 5, that is expressed on neutrophils and other granulocytes [44]. Treatment of human breast cancer xenografts with the combination of doxorubicin, cyclophosphamide and a CXCR2 inhibitor significantly slows tumor growth and metastasis, when compared to chemotherapy or the CXCR2 inhibitor alone [44]. Similarly, docetaxel synergizes with a CXCR2 inhibitor to prevent tumor progression in a GEMM for *Pten*-deficient prostate cancer [45]. In these prostate tumors, infiltrating neutrophils secrete IL1RA to counteract cancer cell senescence and activate proliferation. Clinical support for a role of neutrophils in chemotherapy response comes from observations in a variety of cancer patients, such as breast and non-small cell lung cancer patients, where chemotherapy-induced neutropenia is associated with better patient prognosis [46, 47]. We and others have established a metastasis-promoting role for neutrophils in breast and melanoma models [48-50]. Therefore, targeting neutrophils or their mediators may synergize with chemotherapeutics to specifically decrease metastasis.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature and mature myeloid cells that is predominated by neutrophils with T cell-suppressive functions [51, 52] (**Box 2**). In subcutaneous and orthotopic cell line transplantation models, gemcitabine, 5-FU and doxorubicin directly induce splenic $CD11b^+Gr1^+$ MDSC apoptosis [53-56]. This chemotherapy-induced MDSC death increases the activity of cytotoxic T cells and contributes to tumor control. However, gemcitabine and 5-FU have also been reported to induce activation of the NLRP3 inflammasome in MDSCs, which limits chemotherapy efficacy [56]. Other chemotherapeutics, such as irinotecan, enhance immunosuppression in colorectal cancer patients and in a carcinogen-induced colon cancer model via MDSC expansion [57]. These data indicate that direct killing of MDSCs by chemotherapy can be an additional off-target benefit of anti-cancer therapies, but caution should be exercised, as these effects are chemotherapy- and tumor type-specific.

In a variety of transplantable and chemically induced tumors, specific types of chemotherapy, such as oxiliplatin and anthracyclines, trigger dendritic cell activation through release of HMGB1 and ATP by dying cancer cells [58-60]. This process is called immunogenic cell death and it increases chemotherapy response of certain tumor models through induction of anti-tumor immunity. For many years, it has been thought that dendritic

cells in the tumor bed are immature and lack the ability to prime cytotoxic T cells [61]. However, this notion was recently challenged. *MMTV-PyMT* mammary tumors as well as various transplantable tumors contain two main populations of dendritic cells: $CD11b^{+}CD11c^{+}CD103^{-}BATF3^{-}$ and $CD11b^{-}CD11c^{+}CD103^{+}BATF3^{+}$ cells. The rare $CD11b^{-}$ dendritic cells have superior capability to stimulate cytotoxic T cells [23, 62]; although, intravital imaging has shown that dendritic cells are outcompeted for T cell interaction by TAMs, lessening the chance of a robust anti-tumor immune response [62, 63]. Anti-tumor immunity can be reinstated by combining paclitaxel with TAM depletion or neutralization of their tolerizing abilities (i.e. blocking IL10) [23]. While these studies report that the $CD11b^{-}$ dendritic cell subset is important for chemotherapy response in *MMTV-PyMT* mammary tumors [23, 62], the $CD11b^{+}$ subset plays a greater role in chemotherapy-induced immunogenic cell death of transplantable models [43]. These data emphasize the diverse influence of dendritic cell subsets in chemotherapeutic efficacy. As such, it will be interesting to learn whether the predominant role of one dendritic cell subset is dependent on specific parameters, such as the class of chemotherapy or type of tumor.

Adaptive immune cells

Like innate immune cells, the role of T cells and B cells in chemotherapy response is paradoxical, as these cells may both promote or prevent chemotherapeutic potency. The behavior and function of adaptive immune cells is highly dependent on the class of chemotherapy used and tumor type and stage. Emerging evidence over the last few years has challenged traditionally held views about the anti-cancer contributions of adaptive immune cells. Take antibody-producing B cells, for instance. B cells facilitate squamous cell carcinoma progression in *K14-HPV16* mice through antibody-mediated activation of Fc receptors on TAMs and mast cells, stimulating their pro-angiogenic abilities [64, 65]. B cells also promote squamous cell carcinoma in a carcinogen-induced cancer model [66]. In essence, B cells are viable targets in this tumor type. Indeed, the combination of platinum-based chemotherapy or paclitaxel together with anti-CD20 antibodies in orthotopic squamous cell carcinomas results in stasis of established tumors, whereas chemotherapy or B cell depletion as single agents are completely ineffective [67]. The synergistic effect of chemotherapy and B cell depletion is dependent upon TAMs and $CD8^{+}$ T cells, as depletion of either population desensitizes tumors to the absence of B cells and chemotherapy [67]. Taken together, these studies indicate that inhibiting B cells in combination with chemotherapy may be highly effective for some tumor types.

Studies focused on the role of $CD4^{+}$ T cells provide another example of the complexity surrounding adaptive immune cells in chemotherapy response. One study has shown that $CD4^{+}$ T cells limit the ability of 5-FU to delay the growth of subcutaneous

thymoma cells [56]. 5-FU-exposed MDSCs stimulate $CD4^+$ T cells to express IL17 via IL1β; although, the mechanism by which IL17-producing $CD4^+$ T cells (otherwise known as Th17 cells) counteract the anti-cancer efficacy of 5-FU is unclear. By contrast, IL17 is required for therapeutic efficacy of doxorubicin in a subcutaneous sarcoma model, and γδ T cells, not $CD4^+$ T cells, are the source of IL17 in this scenario [68]. In *MMTV-Neu* mice – a model driven by wild-type rat ERBB2 [69] – inhibition of the immunosuppressive enzyme indolamine 2,3-dioxygenase (IDO), cooperates with cisplatin, cyclophosphamide, doxorubicin and paclitaxel to retard tumor growth [70]. Interestingly, the anti-proliferative effects of IDO inhibition and paclitaxel are dependent on $CD4^+$ T cells, as their depletion reverses the phenotype. *MMTV-Neu* cell lines injected into nude mice and treated with paclitaxel/IDO inhibitor phenocopy the $CD4^+$ T cell depletion experiments in the *de novo* tumor model [70]. Conversely, $CD4^+$ T cell depletion further delays *MMTV-Neu* tumor growth in mice treated with doxorubicin and lapatinib – a small molecule inhibitor of EGFR and ERBB2 [71]. The importance of $CD4^+$ cells following paclitaxel or doxorubicin, without the addition of IDO inhibtors or lapatinib, remains to be established in mammary tumor-bearing *MMTV-Neu* mice, since these controls were not included in either study [70, 71]. Nonetheless, manipulation of Th17 cells or other $CD4^+$ T cell subsets may be a useful strategy to combat cancer growth and metastasis when used in combination with chemotherapy.

FOXP3-expressing regulatory cells (Tregs) are the most notorious subpopulation of $CD4^+$ T cells, known for their ability to suppress anti-tumor immune responses [72]. As early as the 1980s, it was recognized that Tregs in tumor-bearing mice are sensitive to cyclophosphamide [73], and more recent studies confirmed this both in non-tumor- and tumor-bearing rodent models [74, 75]. The depletion of Tregs using anti-CD25 antibodies synergizes with other types of chemotherapy, including platinum-containing agents and etoposide, to reduce tumor growth in subcutaneous, transplantable models [76, 77], as well as a GEMM of lung adenocarcinoma [78]. The mechanism of synergy is most likely dependent on reactivation or re-infiltration of $CD8^+$ T cells in tumors [77]; however, this remains to be confirmed.

Cytotoxic lymphocytes, including $CD8^+$ T cells and natural killer (NK) cells, have been reported to contribute to the efficacy of certain chemotherapeutics. For example, depletion of NK cells abolishes the tumor-shrinking ability of cyclophosphamide in tumor-bearing immunodeficient mice [42] and in an experimental melanoma metastasis model [79]. $CD8^+$ T cells with anti-tumor activity are unleashed upon treatment with a non-cytotoxic dose of paclitaxel in a spontaneous melanoma GEMM [80]. Similarly, $CD8^+$ T cells contribute to cancer cell killing by immunogenic cell death-inducing chemotherapeutics in a variety of transplantable and carcinogen-induced tumors [59, 60, 81]. Chemotherapy-driven

immunogenic cell death is not dependent on NK cells [60], but IFN signaling is important in this process [82].

Recently, we and others have challenged the currently held dogma that cytotoxic $CD8^+$ T lymphocytes are required for tumor regression following specific chemotherapeutic agents [59, 60, 81]. For these studies, we used two different GEMMs of breast cancer: *K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice, a model for invasive lobular breast cancer driven by the stochastic loss of E-cadherin and p53 [83]; and *MMTV-NeuT* mice, a model driven by a mutated form of the rat proto-oncogene ERBB2 [84]. We showed that the adaptive immune system is dispensable for response to oxaliplatin, doxorubicin and cisplatin [85]. In line with these data, depletion of $CD8^+$ T cells in *MMTV-PyMT* mammary tumor-bearing mice fails to counteract the efficacy of paclitaxel [22, 23], indicating that $CD8^+$ T cells are also dispensable in this experimental setting. Additionally, $CD8^+$ T cell depletion in combination with 5-FU treatment of subcutaneous EL4 thymomas has no impact on tumor growth [56]. Taken together, these observations underscore the plasticity within the adaptive immune system in response to different chemotherapeutic regimens and suggest that chemotherapy on its own may not be enough to elicit anti-tumor immune responses in spontaneous epithelial tumors. Chemotherapy together with additional anti-cancer agents, such as targeted therapies and immunosuppression inhibitors, may be required to fully reactivate cytotoxic T lymphocytes.

Influence of the immune system on radiotherapy

Approximately 50-60% of all cancer patients are treated with radiotherapy and this regimen is given alone or in combination with chemotherapy and/or surgery [86, 87]. Ionizing radiation induces DNA damage in the form of single-strand and double-strand breaks. As a consequence, a number of cellular events can occur including DNA damage recognition, cell cycle checkpoint activation, DNA repair and/or apoptosis pathways. Dying cells then release stress proteins and other factors that can be sensed by various immune cells to clear away cellular debris, initiate tumor recovery processes or secondary anti-cancer responses. The participation of innate and adaptive immune cells in radiotherapy efficacy is discussed here.

Innate immune cells

Several studies have reported increased recruitment of monocytes and macrophages following irradiation of tumor-bearing mice [88-93]; although, the similarity to radiotherapy-treated human tumors needs further investigation. In mice, radiotherapy-induced TAM infiltration is mainly attributed to radiation-induced hypoxia and the subsequent surge in hypoxia-regulated chemokines, such as CXCL12 [90, 93]. Monocytes and macrophages expressing TIE2 – the receptor for Angiopoietins 1 and 2 – are highly receptive to increased hypoxia and CXCL12 levels [90, 93, 94], and TIE2-expressing monocytes/macrophages

have a profound ability to counteract hypoxia through the induction of angiogenesis [95]. As one may predict, neutralizing CXCL12 or blocking its receptor, CXCR4, to prevent TAM accumulation further delays tumor progression when combined with radiotherapy in orthotopic syngeneic and xenograft models of glioblastoma [90], as well as subcutaneous xenografts of lung carcinoma and syngeneic mammary tumors [93].

When TAMs are depleted in various subcutaneous transplantable and xenograft models by targeting all $CD11b^+$ cells, the inhibitory effects of radiation on tumor growth and angiogenesis are augmented [88, 89]. This result may be largely explained by the contribution of MMP9 by $CD11b^+$ cells that drives tumor regrowth through vasculogenesis [88, 89]. Similar results are observed using other strategies to block TAMs, including CSF1R inhibitors in combination with fractionated irradiation in subcutaneous prostate tumors [91] and carrageenan in transplantable models [92]. In B16 melanomas, however, the anti-cancer effect of a single local high radiotherapy dose is not affected by the absence of TAMs [96]. Depletion of $Ly6G^+$ neutrophils and $Ly6C^+$ inflammatory monocytes using Gr1 antibodies has no synergistic effect with radiotherapy in subcutaneous human prostate tumors [89]. Conversely, the depletion of neutrophilic MDSCs potentiates the efficacy of radiotherapy on subcutaneous colon cancer cells [97], presumably through the alleviation of T cell suppression. Dendritic cells also play a role in radiosensitivity [58, 96, 98], but their activation by irradiation varies between transplantable models. For example, HMGB1-sensing $TLR4^+$ dendritic cells are required for radiotherapy efficacy in subcutaneous thymomas [58]. By contrast, inhibition of HMGB1 or knockout of downstream TLR4 signaling components has no effect on subcutaneous colon cancer cells following radiotherapy [99]. In this model, radiotherapy response is dependent on type I IFN signaling in dendritic cells and the adaptor protein STING [98, 99]. Whether the discrepancy between the roles of myeloid cells in these studies is caused by the differences in tumor model, or by the differences in radiotherapy dose and schedule remains to be investigated. What is also absent from this area of anti-cancer therapy research is how myeloid cells respond to irradiated metastases in mouse models.

Adaptive immune cells

Various independent research groups have reported that radiosensitivity requires $CD8^+$ T cells for tumor control in transplantable models [58, 96, 99-101]. On the other hand, $CD4^+$ T cells may not be so important for this process [96]. Similar experiments in GEMMs are unavailable, so the importance of $CD8^+$ T cells in radiotherapy response remains unanswered in these models. Interestingly, one study showed that paclitaxel together with irradiation actually increases mammary tumor growth and pulmonary metastases when compared to irradiation alone [100]. The same study switched to a different model system to

explain this phenomenon. Experiments using dacarbazine and radiotherapy in B16 melanomas showed that the radiation-induced priming and activation of $CD8^+$ T cells is blunted by chemotherapy [100]. Whether the combination of chemotherapy and radiotherapy is detrimental to T cell priming in cancer patients is unclear at present. Based on these data, enhancement of $CD8^+$ T cell activity in combination with radiotherapy may provide additional benefit to cancer patients. Indeed, mice bearing transplantable *MMTV-PyMT* mammary tumors depleted of Tregs and treated with irradiation survive longer than either radiotherapy or Treg depletion alone [102]. Various immunotherapeutic strategies to achieve these effects will be discussed below.

Contribution of immune cells to targeted therapy

Over the past decade, targeted therapies have emerged from the identification of tumor type-specific driver mutations and hyperactive signaling pathways. Some examples include BRAF inhibitors (vemurafenib) for melanomas, ERRB2 inhibitors (trastuzumab) for $HER2^+$ breast cancer, and PARP inhibitors (olaparib) for BRCA-deficient breast and ovarian tumors (**Figure 1**). Many of these are performing exceptionally well in the clinic. Yet, the lack of durable responses is posing a major problem highlighting the need to find synergistic therapies. The importance of stromal cells in mediating resistance to targeted therapies has recently been shown *in vitro* using an extensive co-culture system. In this study, fibroblasts were reported to secrete HGF that activates the MET receptor in melanoma cells and downstream MAPK and AKT signaling pathways to bypass the dependency on BRAF [103]. Here, we highlight the studies pertaining to immune cells and mouse models. A more extensive discussion has been provided elsewhere [104].

When assessing the influence of innate and adaptive immune cells on the efficacy of targeted therapies, it is important to take into account the distinctive properties of the two types of targeted drugs: monoclonal antibodies and small-molecule inhibitors. Unlike small-molecule inhibitors, therapeutic antibodies can activate immune cells, such as macrophages, neutrophils and natural killer cells, via binding to their Fc receptors, resulting in complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC) [105]. Thus, the actual working mechanism of the targeted antibody drugs is in part dependent on their ability to trigger immune cell activation, whereas this is not the case for small-molecule inhibitors.

Innate immune cells

In mice bearing melanoma cell lines derived from *Braf^{V600E};Tyr::CreERT2* tumors, the release of TNF by TAMs protects tumors from MEK inhibitor-induced cell death [106]. This resistance mechanism can be overcome by combining MEK inhibitors with an inhibitor of NF-κB

signaling to prevent TAM accumulation and TNF secretion in tumors. Interestingly, TNF expression is independent of the state of TAM polarization in this model, as all cultured macrophages expressed TNF regardless of the stimuli used *in vitro* to skew their polarization [106]. By contrast, TAM polarization may be important for the response to targeted therapies in other cancer types and can even result in adverse effects. Imatinib treatment of tumor-bearing $Kit^{V558/+}$ mice – which carry a gain-of-function point mutation on one allele of the *Kit* receptor gene predisposing them to spontaneous gastrointestinal stromal tumor (GIST) development [107] – has been shown to repolarize TAMs from their normal anti-tumorigenic, T cell-stimulating phenotype to a more pro-tumorigenic phenotype [108]. However, the consequence of TAM skewing by imatinib, whether this is beneficial or detrimental for tumor progression, remains untested.

Numerous studies have shown that Fc receptor (FcR) expression on TAMs and neutrophils is required for the response to antibody-based targeted therapies through ADCC. Using various transplantable models in knockout mice that lack one or more FcRs, tumor regression mediated by rituximab, anti-CTLA4, trastuzumab and other anti-ERBB2 antibodies is reversed [109-115]. A lymphoma patient study showing that high TAM infiltration correlates with improved prognosis after a rituximab-containing regimen, but worsened prognosis without rituximab, supports these observations [116]. These data suggest that whereas high numbers of TAMs serve as an indicator of poor disease outcome in untreated, chemo- or radiotherapy treated patients, they may predict good disease outcome in patients treated with targeted antibody drugs. Of note, however, FcR activation on TAMs and mast cells by endogenous antibodies promotes squamous cell carcinoma progression and pro-tumorigenic myeloid cell polarization in the *K14-HPV16* model [65]. Similarly, the therapeutic antibody targeting EGFR, cetuximab, induces an immunosuppressive phenotype in human monocytes cultured with colon cancer cell lines *in vitro* [117]. These data suggest that the importance of FcR expression on myeloid cells in regulating therapeutic antibody response may be context, drug and model dependent.

It has been reported that the efficacy of anti-ERBB2 antibodies is also dependent on HMGB1 and TLR signaling [110], suggesting that the mechanism of targeted-therapy induced tumor regression may be similar to immunogenic cell death processes. However, the addition of doxorubicin, an immunogenic cell death-inducing chemotherapy, to anti-ERBB2 treatment counteracts the effects of single agent anti-ERBB2 and fails to augment the rejection of established mammary tumors. Why the combination of two immunogenic cell death inducers fails to synergize remains a mystery. Paclitaxel, on the other hand, boosts the effects of anti-ERBB2 treatment and this combination results in tumor rejection in 100% of mice [110]. These data underscore the importance of optimally matching targeted therapy with chemotherapeutic agents.

There is evidence from pre-clinical models that targeting the JAK/STAT pathway counteracts immunosuppression and controls cancer progression. For example, *Pten*-deficient prostate tumors from *Probasin-Cre4;Pten*$^{F/F}$ mice exhibit activation of the JAK2/STAT3 pathway that mediates a MDSC-driven immunosuppressive environment. Genetic deletion of *Stat3* in prostate epithelial cells or treatment of prostate tumors with a JAK2 inhibitor in combination with docetaxel prevents MDSC recruitment to tumors and slows tumor growth [118]. In addition, JAK2/STAT3 inhibition in mice bearing subcutaneous sarcomas that lack STAT3 activation modulates MDSC and dendritic cell proportions as well as their activity to reinstate anti-tumor immunity [119]. Since JAK/STAT inhibition directly affects immune cell abundance and phenotype in some models, it is tempting to speculate about the implications beyond this study. Targeted therapies that take out two birds with one stone – cancer cells and immunosuppressive myeloid cells – could result in more positive outcomes than when two distinct anti-cancer therapies are used simultaneously.

Adaptive immune cells

Elegant proof-of-principle experiments performed in transgenic mouse models, where targeted therapy is emulated by switching off an oncogene-driving mutation (i.e. MYC) during tumor development, have shown that T cells mediate tumor clearance through the killing of both cancer cells and endothelial cells [120, 121]. These studies established the importance of T cells in mouse models that mimic targeted therapies, and there are other reports demonstrating the importance of adaptive immune cells using specific targeted therapies. Treatment of melanoma patients with BRAF inhibitors increases infiltration of $CD4^+$ and $CD8^+$ T cells into tumors and this correlates with reduced tumor size [122]. In experimental melanoma metastasis models, NK cells mediate the anti-tumor effects of a BRAF inhibitor, while $CD4^+$ and $CD8^+$ T cells are dispensable [123]. By contrast, $CD8^+$ T cells are required for the response of BRAF inhibitors in transplantable melanoma models [124, 125], $CD4^+$ T cells, but not $CD8^+$ T cells, mediate tumor clearance following BRAF inhibitor treatment of spontaneous melanomas in a GEMM [126] (*Braf*^{V600E}*;Pten*$^{F/F}$*;Tyr::CreERT2* mice [127]). Interestingly, each of these examples using melanoma models applied the same BRAF inhibitor, PLX4720, which is a research analogue of vemurafenib. But why does each study show a dependency on a different immune cell population? One explanation may be the location of the tumor in the models used, including subcutaneous, skin and lungs, as well as the timing of targeted therapy in relation to immune cell depletion. Similarly, imatinib efficacy is dependent on NK cells in melanoma metastasis models [128], whereas $CD8^+$ T cells contribute to tumor regression following imatinib treatment of GIST-bearing mice or dasatinib treatment of subcutaneous mastocytoma-bearing mice [129, 130].

In terms of immune regulation, mTOR inhibitors are very interesting targeted drugs. mTOR is a critical regulator of immune function, as it promotes differentiation, activation and function of T cells, B cells and antigen-presenting cells [131]. It also controls the balance between effector T cells and Tregs [132]. Based on its strong immunomodulatory effects, mTOR inhibition has been successfully utilized to prevent transplant rejection over the last decades. In the cancer setting, the immunosuppressive effects of mTOR inhibitors are very complex and mTOR inhibitor dependent. For example, the mTOR inhibitor AZD8055, in contrast to rapamycin, enhances the anti-cancer efficacy of an CD40 agonist by activating TAMs and DCs and induces a strong Th1 response in an experimental liver metastasis model [133]. Similarly, temsirolimus synergizes with depletion of $CD4^+$ T cells or Tregs to reactivate $CD8^+$ T cells and reduce the growth of subcutaneous renal cell carcinomas [134].

Further investigations should work out whether dependency of targeted therapies on adaptive immune cells is tumor type and/or location specific. If indeed the importance of adaptive immune cells in regulating the response to targeted therapy is governed by the location of the tumor, these data would suggest that the role of immune cells are also likely to be different between primary tumors and metastasis in distant organs.

Immune cell function following vascular-targeting agents

Angoigenesis inhibitors – the most famous being the anti-VEGF antibody, bevacizumab – and vascular damaging agents target blood vessels and thus limit re-oxygenation and delivery of nutrients (**Figure 1**). The link between angiogenesis and the immune system is well established [135], so perhaps it is not surprising that immune cells regulate the response to anti-angiogenic therapies.

The pro-angiogenic functions of TAMs have been known for about a decade [95, 136, 137]. More recent studies have shown that TAMs are recruited to experimental tumors following different forms of anti-angiogenic therapies [138-140], often because of the hypoxia-induced increase in chemotactic factors [94, 138]. Various studies have reported that TAMs counteract the efficacy of anti-angiogenic agents. For example, TAM depletion with clodronate liposomes synergizes with sorafinib in human hepatocellular carcinoma xenograft models [141] and with the anti-VEGFR2 antibody, DC101, in subcutaneous colon tumors [142] to reduce tumor growth. Synergy also occurs when combining a CSF1R inhibitor with DC101 in another transplantable model [143].

Blockade of the Angiopoietin-TIE2 signaling axis is another potent strategy to prevent tumor angiogenesis and slow tumor growth. In *MMTV-PyMT* mice, an anti-Angiopoietin 2 (ANGPT2) antibody not only decreases blood vessel density and retards tumor progression, but it also prohibits TIE2-expressing macrophages from associating with endothelial cells. The TAM-endothelial cell interaction is required for angiogenesis, since conditional deletion

of *Tie2* in TAMs decreases blood vessel density and mirrors anti-ANGPT2 treatment [140]. In addition, ANGPT2 inhibitors reduce lung metastasis in spontaneous breast cancer metastasis models [140, 144]. The effect of ANGPT2 inhibitors on metastasis most likely occurs during the late stages of the metastatic cascade when monocyte-derived macrophages facilitate angiogenesis [145], since neutralization of ANGPT2 decreases CCL2-dependent monocyte recruitment to lungs and ICAM-mediated monocyte adhesion to endothelial cells [144]. Furthermore, inhibition of recruitment of TIE2-expressing macrophage to transplanted tumors via CXCR4 blockade amplifies the tumor inhibitory effect of the vascular-damaging agent, combretastatin, indicating that this subset of TAMs counteracts the efficacy of combretastatin [138]. Thus, combining inhibitors of both TAMs and the Angiopoietin-TIE2 axis may yield promising tumor-reductive results.

Studies from a few years ago showed that tumor-induced $CD11b^+Gr1^+$ cells (**Box 2**) also mediate intrinsic resistance to anti-VEGF therapies [146]. More recently, a suppressive functionality was demonstrated for these cells [147], indicating that $CD11b^+Gr1^+$ cells in this subcutaneous lymphoma model can be categorized as MDSCs. These $CD11b^+Gr1^+$ cells express pro-angiogenic molecules, like PROK2/BV8, that circumvent the dependency of transplantable tumors on VEGF. Targeting MDSCs or PROK2/BV8 synergizes with anti-VEGF treatment to reduce tumor growth [146, 148, 149]. Tumor-derived G-CSF is responsible for initiating this cascade by mobilizing MDSCs and up-regulating their expression of PROK2/BV8. As one may predict, neutralization of G-CSF also synergizes with anti-VEGF therapy [149]. The cytokine, IL17, is also involved in this cascade. Like inhibition of MDSCs, PROK2/BV8 or G-CSF, blockade and genetic knockout of IL17 sensitizes resistant, transplanted tumors to anti-VEGF therapy [147]. Interestingly, $CD4^+$ T cells appear to be the source of IL17 in these tumor models and IL17 regulates G-CSF expression in tumor-associated fibroblasts. We have recently shown that IL17-producing $\gamma\delta$ T cells induce systemic expression of G-CSF to expand immunosuppressive neutrophils and facilitate breast cancer metastasis [49]. In this regard, targeting the IL17-producing T cell—G-CSF—neutrophil axis in combination with anti-angiogenic therapies may benefit patients with metastatic disease.

Much less is known about the role of Tregs and T cells during anti-angiogenic therapy. In renal cell and colorectal carcinoma patients, sunitinib reduces the number of Tregs and MDSCs [150-152] and bevacizumab does the same in colorectal carcinoma patients [151]. A few experimental studies have shown that endogenous T cell infiltration is increased following anti-angiogenic agents [153-155], and one study has shown that the efficacy of DC101 treatment is dependent on both $CD4^+$ and $CD8^+$ T cells in a transplantable mammary tumor model [153]. As such, the need for more investigations into the role of

immunosuppressive and adaptive immune cells is warranted to help guide the future clinical possibility of combining vascular-targeting agents with immunotherapy.

Immunotherapeutic strategies to enhance the response to anti-cancer therapies

The studies highlighted above have established that both innate and adaptive immune cells are viable targets for therapeutic manipulation. Three main immunomodulatory approaches are under intense pre-clinical and clinical investigation: 1) immunotherapy aimed at boosting the patients' own immune system to fight cancer, for example via T cell checkpoint inhibitors targeting CTLA4 and the PD1-PDL1 axis (**Figure 1**), or via cancer vaccines; 2) immunotherapy through adoptive transfer of (genetically engineered) autologous T cells; and 3) therapies aimed at suppressing pro-tumor inflammatory processes (**Figure 1**), such as anti-CSF1R [7] and anti-CCL2 [156, 157].

The clinical success of immunotherapeutics that block negative immune regulatory pathways, including anti-CTLA4 (ipilimumab) and anti-PD1 (pembrolizumab and nivolumab) [158-160], has reinvigorated cancer research and oncology. The potential of these drugs shows no signs of stopping, since the list of tumor types that respond to checkpoint inhibitors is expanding rapidly [161, 162]. However, checkpoint inhibitors do not benefit every patient [158, 163, 164]. To increase the number of cancer patients that benefit from immunotherapy, it will be critical to fill in several gaps. First, biomarkers that preselect those patients most likely to respond to immunotherapy need to be uncovered and implemented into clinical practice. Recent reports suggest that both mutational load as well as the nature of neo-antigens might dictate whether a tumor will respond to immune checkpoint inhibitors [165]. Additionally, high intratumoral $CD8^+$ T cells, $PD1^+$ and $PDL1^+$ cells are associated with increased responsiveness to therapies targeting PD1-PDL1 signaling [160-162, 166]. Since many human cancers are characterized by the influx of T cell-suppressive immune cells, such as Tregs, TAMs, MDSCs and neutrophils, it is likely that the quantity and/or phenotype of these cells also contains predictive value. Second, the most efficacious combinations of conventional anti-cancer therapies and immunotherapy need to be established. Given the predictive power of $CD8^+$ T cell infiltration in tumors, anti-cancer therapies that augment $CD8^+$ T cell infiltration and inhibit immunosuppressive cells into tumors are obvious candidates to test with immune checkpoint inhibitors. In support of this notion, gemcitabine – a chemotherapeutic that targets Tregs and MDSCs [53, 167, 168] – and melphalan synergize with anti-CTLA4 in transplantable tumor models [169, 170]. Experimental and clinical studies have also revealed synergy between cyclophosphamide – known for its Treg-reducing effects [73-75] – and various immunotherapeutic approaches [171, 172]. By contrast, the synergy between cisplatin, a chemotherapeutic that does not affect immunosuppressive cells, and anti-CTLA4 is controversial [170, 173]. Radiotherapy also synergizes with CTLA4, PDL1

and combined CD40/CD137 targeting to control the growth of various transplantable models and lung metastases [97, 174, 175] – a process that is dependent on $CD4^+$ T cells, $CD8^+$ T cells, NK cells or all of the above [97, 174]. These data suggest that intervention strategies that induce a favorable T cell influx and/or reduced Foxp3/CD8 ratio in tumors are efficacious partners for checkpoint inhibitors.

Chemo-, radio-, targeted and anti-angiogenic therapies have all been shown to increase recruitment of adoptively transferred T cells to transplanted tumors and enhance anti-tumor responses [96, 176-183], but the mechanism by which this occurs has not been fully elucidated. One recent report showed that irradiation of spontaneous pancreatic tumors in RIP1-Tag5 mice increases recruitment of adoptively transferred $CD8^+$ T cells and T cell-mediated tumor rejection, which depended on repolarization of TAMs towards an anti-tumorigenic phenotype [184]. Thus, strategies that condition the microenvironment to become more receptive to T cells with anti-tumor activity may enhance tumor eradication. Intriguingly, the tumor microenvironment not only regulates the initial therapeutic effect of adoptively transferred T cells by influencing their intra-tumoral recruitment, but can also induce resistance of tumors to adoptively transferred T cells. In a GEMM of melanoma, cancer cells acquire resistance to T cell adoptive transfer through inflammation-induced tumor cell dedifferentiation, which is characterized by reversible loss of tumor antigen expression [185].

As discussed above, many tumors are characterized by influx of myeloid cells with immunosuppressive activity, such as macrophages, MDSCs and neutrophils, which may impede successful T cell-mediated eradiation of cancer cells. Relieving the immunosuppressive networks in tumor-bearing patients might be an alternative strategy to maximize success of immunotherapy and there is experimental evidence to support this idea. For instance, blockade of CXCR2-mediated MDSC trafficking into transplanted rhabdomyosarcomas increases the efficacy of anti-PD1 therapy [186]. In an orthotopic pancreatic tumor model, the triple combination of gemcitabine, TAM blockade via CSF1R inhibition and anti-CTLA4 or the quadruple combination with anti-PD1 is most effective at inducing tumor regression [35]. Blockade of CCL2, a monocyte chemoattractant, has been reported to increase the anti-cancer efficacy of various cancer vaccines on subcutaneous tumors [187]. A growing number of next-generation immunomodulatory drugs aimed at targeting tumor-associated myeloid cells is being developed and tested in clinical trials [7]. Combining these immunomodulatory drugs with the emerging immunotherapeutic approaches will likely increase the number of cancer patients that benefit from immunotherapy.

Concluding remarks

What is clear from the aforementioned experimental and clinical studies is that the immune system is a major regulator of anti-cancer therapy response and resistance. At the same time, it is difficult to deduce one over-arching conclusion from these studies, because of the overwhelming complexity and the diversity of immune cell responses to specific anti-cancer therapies. What we can say for sure is that the involvement of immune cells is largely dictated by tumor type, mutational signature, tumor model and tumor location (e.g. orthotopic vs. subcutaneous) (**Table 1**), and generalizing immune cell response to a particular anti-cancer therapy across multiple tumor types or locations should be avoided. Proof of this principle was recently provided by directly comparing the efficacy of immunotherapy on three orthotopic tumors versus their subcutaneous counterparts. This study showed that in addition to microenvironmental differences in immune cell profile and vascularity, orthotopic tumors are more immunosuppressive in nature and less sensitive to immunotherapy than subcutaneous tumors [188]. We have also learned that conventional anti-cancer therapies have both direct and indirect effects on the immune system (**Figure 2**). Relatively little is known about the role of immune cells during other anti-cancer therapies not mentioned here, such as hormonal therapy. Future experiments and clinical trials will undoubtedly broaden our knowledge in this arena.

Right now, the excitement and success of immune checkpoint inhibitors in advanced cancer patients has set the stage for new therapeutic approaches in the treatment of cancer with a focus on combined targeting of cancer cell-intrinsic and -extrinsic processes. For optimal clinical implementation, however, a number of key questions and issues need to be addressed (**Box 4**). First, conventional therapies need to be optimally matched to specific immunotherapy and/or immunomodulatory drugs to achieve maximal benefit. Timing of treatments will be crucial in this process. These combinations will require certain aspects of personalization, taking into account tumor type, mutation status and intra-tumoral immune profiles prior to treatment. Second, researchers should be on the lookout for immune-based resistance mechanisms limiting the efficacy of traditional anti-cancer therapies and immunotherapies. Since immune cells are highly versatile and plastic cells that are designed to adapt quickly to a variety of unanticipated situations, resistance to immunotherapy and immunomodulatory agents is inevitable and may be dictated by rewiring of immune processes. Finally, more effort should be focused on metastasis. Metastatic disease, not primary tumors, is responsible for the majority of cancer-related mortality and controlling metastatic disease is the most urgent need in the clinic. The discrepancy between the effects of anti-cancer therapies on experimental tumor growth and the response to these drugs in metastatic patients enrolled in early clinical trials may explain why about 85% of new drugs fail [189]. Mouse models have revealed meaningful disparities between the response of

primary tumors and their visceral metastases to anti-cancer therapies [190, 191]. In this regard, choosing pre-clinical models that accurately represent each stage of the metastatic cascade is also critical to understand basic biological mechanisms of metastasis formation as it occurs in patients [192] and to specifically target metastatic disease (**Figure 3**). Furthermore, targeting both cancer cells and immune cells may be the key to prevent metastasis from occurring and to combat established metastasis.

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Box Legends

Box 1: Comparison of mouse models used in anti-cancer therapy research. In immune-related anti-cancer therapy research, three types of mouse models are commonly used including cancer cell line-based transplantable, carcinogen-induced and genetically engineered mouse models (GEMMs) of cancer. Several advantages and disadvantages are presented for each model. Transplantable models rely on the injection of *in vitro* cultured cancer cells into recipient immunocompetent or immunodeficient mice and this is usually done subcutaneously. In carcinogen-induced models, chemical carcinogens are injected into, or topically applied to mice to induce tumors, where the type and location of carcinogen dictates the location of the tumors formed. For example, topical application of DMBA/TPA results in skin carcinogenesis, and injection of methylcholanthrene (MCA) intramuscularly results in fibrosarcomas. One caveat of this model is that tumors do not always form in carcinogen-injected mice. By their nature, carcinogen-induced tumors represent only a small fraction of human cancers. GEMMs are driven by specific mutations in oncogenes or tumor suppressors. The first generation GEMMs were developed in the 1980s and depended on germ-line introduction of oncogenes, whose constitutive expression could be spatially controlled by tissue-specific promoters. Many of the experimental studies addressing the causal link between immune system, tumorigenesis and therapy efficacy have used these so-called ‘onco-mice’. An example is the *MMTV-PyMT* mouse model for breast cancer, in which the Mouse Mammary Tumor Virus (MMTV) promoter drives a viral oncogene, Polyoma Middle T antigen. *MMTV-PyMT* mice develop multifocal tumors in all five pairs of mammary glands, as well as spontaneous lung metastases [193]. To overcome some of the drawbacks

of the first-generation GEMMs – such as embryonic lethality or the development of tumors outside the tissue of interest – methods have been developed to conditionally induce somatic mutations in a tissue-specific and/or time-dependent manner. An example of a second-generation GEMM is the *K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* model in which stochastic Cre recombinase-mediated loss of the floxed genes encoding for E-cadherin and p53 results in the formation of mammary tumors resembling human invasive lobular breast cancer [83].

Box 2: Immune complexity in tumor-bearing hosts

The immune system has long been postulated to protect against cancer and metastatic spread; yet, tumors exploit a number of strategies to successfully evade destruction by the immune system. Cancer cells hijack the immune system for their own benefit, allowing themselves to escape from immune attack, maintain limitless proliferation, survive under dire circumstances and spread to distant organs. As such, immune cells and their inflammatory mediators can create a hospitable microenvironment that is favorable for cancer outgrowth [194].

Immune cells and their mediators are abundantly present in the microenvironment of (disseminated) cancer cells. The exact nature of the tumor-induced local and systemic immune alterations is dictated by the genetic make-up of the tumor (*i.e.* type of oncogenes/loss of tumor suppressors, mutational load), tumor type (tissue of origin and etiology), tumor stage, therapy history and age of the patient.

Macrophages and neutrophils make up a significant proportion of the inflammatory infiltrate in many tumors and their accumulation in cancer patients has been associated with poor prognosis [22, 195, 196]. Experimental studies have confirmed pro-tumor and pro-metastatic functions for these tumor-associated myeloid cells [197]. Another type of tumor-associated immune cell that has gained a lot of recent attention is the myeloid-derived suppressor cell (MDSC) [51, 52]. MDSCs represent a heterogeneous group of immature $CD11b^+Gr1^+$ cells that includes precursors of macrophages, granulocytes and dendritic cells at different stages of their differentiation, and they are defined by their functional ability to suppress T cell proliferation. Tumor-derived mediators promote aberrant differentiation of myeloid lineage cells, resulting in accumulation of MDSCs in the circulation and lymphoid organs. MDSCs are potent immunosuppressive and pro-angiogenic cells and their accumulation in the circulation of solid cancer patients has been linked with disease progression and metastasis [198-200].

Alongside myeloid cells, adaptive immune cells are frequently found in tumors. Their role in tumorigenesis is rather paradoxical [201]. Whereas $CD8^+$ T cells potentially recognize and kill tumor cells, $CD4^+$ T cells, Tregs, B cells and $\gamma\delta$ T cells play a more sinister role in tumor biology [49, 64, 65, 78, 202]. In tumor-bearing hosts, crosstalk between adaptive and

innate immune cells fosters disease progression. Tumor-associated myeloid cells frequently suppress $CD8^+$ T cells and induce Tregs, while at the same time, cells of the adaptive immune system, notably B lymphocytes, $CD4^+$ T cells and $\gamma\delta$ T cells, can actually contribute to the expansion and pro-tumor polarization of myeloid cells in tumor bearing hosts [49, 64, 65, 78, 202].

Box 3: Immune cell polarization

As normal epithelial cells make the transition to cancer cells, they induce the aberrant expression of molecules whose concentration is unphysiological, or molecules that may be entirely new to a particular tumor-originating location. Immune cells respond to these mutation-driven cues both locally and systemically and the resulting effect is a skewing of their phenotype and behavior. In addition to cancer cell-derived factors, physiological aspects of the tumor microenvironment, such as hypoxia and pH, and factors from other cell types also educate immune cells. This alteration in immune cell appearance and function is referred to as polarization. For many years, researchers have used a binary nomenclature that reflects extreme ends of immune cell polarization. As an example, macrophages are often referred to as pro-tumorigenic M2 TAMs or anti-tumorigenic M1 TAMs. This has led to the misconception that there are only two types of TAMs. Recent gene expression data from several independent laboratories has discredited this oversimplified idea, showing that TAMs comprise several distinct populations and share properties of both M1 and M2 cells [203-205]. In regards to therapy, repolarization of immune cells from a pro-tumorigenic state to a more anti-tumorigenic is one strategy that may enhance the efficacy of traditional anti-cancer therapies.

Box 4: Outstanding questions

- What are the determinants in each tissue/tumor type that dictate the involvement of immune cells to therapy response?
- What is the role of other, more rare populations of immune cells – like mast cells, eosinophils and innate lymphoid cells – in anti-cancer therapy response and resistance?
- Which tumor types will be most responsive to immunotherapy and/or immunomodulatory agents, and what are the underlying mechanisms?
- How can the most optimal therapy combinations be determined for individual patients?
- What are the mechanisms underlying resistance towards immunotherapy and immunomodulatory agents, and how can resistance be anticipated and prevented?

- Should immune-based treatment strategies for metastatic patients be the same as non-metastatic patients?
- How can GEMMs be further sophisticated to better model anti-cancer therapy response in humans?

Glossary Box

Alkylating agents: A class of chemotherapy drugs that directly damage DNA by substituting alkyl groups for hydrogen atoms on DNA, causing the formation of cross links within DNA chains and thereby resulting in cell death. Examples of alkylating agents are cyclophosphamide and melphalan.

Anthracyclines: A class of chemotherapy drugs that is widely used to treat many different types of cancer. Anthracyclines prevent cell division by disrupting the structure of the DNA via several mechanisms. Examples of anthracyclines are doxorubicin and daunorubicin.

Braf^{V600E};Tyr::CreERT2 **or** ***Braf^{V600E};Pten$^{F/F}$;Tyr::CreERT2*** **mouse tumor models*:*** A conditional GEMM of melanoma driven by an activated form of BRAF and loss of PTEN under the control of the *Tyrosinase* promoter. Tumors are induced by topical administration of tamoxifen to the skin, so timing of tumor development can be initiated as desired.

C3(1)-Tag **mouse tumor model*:*** A GEMM model in which SV40 large T antigen expression under the control of the 5' flanking region of the C3(1) component of the rat prostate steroid binding protein drives tumor development. In females, mammary ductal epithelium is transformed leading to invasive mammary tumors that resemble human ductal carcinoma *in situ* (DCIS). Male mice develop phenotypic changes in the prostate that progress into invasive carcinoma.

Genetically engineered mouse model (GEMM) for cancer: In GEMMs for cancer, normal cells are transformed *in situ* as a consequence of germline or somatic mutations in specific cell types, resulting in the development of spontaneous tumors that faithfully recapitulate each stage of cancer progression – from tumor initiation to advanced disease and in some models also metastasis.

K14-HPV16 **mouse tumor model**: A GEMM for *de novo* squamous carcinogenesis of the skin. These mice transgenically express the early region genes of the human papilloma-virus type 16 (HPV16) under control of the human keratin 14 promoter/enhancer. Cervical tumors

can also be induced in these mice by administration of low-dose estrogen, hence K14-HPV16/E_2.

***K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mouse tumor model:** A conditional GEMM for invasive lobular breast cancer. These mice transgenically express cre-recombinase under control of the human keratin 14 promoter. In these mice, the alleles encoding for E-cadherin and p53 are homozygously floxed. As a consequence, mammary and skin epithelial cells stochastically lose E-cadherin and p53, which induces the formation of tumors in these tissues.

***Kit*$^{V558/+}$ mouse tumor model**: These mice carry a gain-of-function point mutation on one allele of the *Kit* receptor gene predisposing them to spontaneous gastrointestinal stromal tumor (GIST) development.

Metastatic Cascade: Cancer dissemination is a multistep process, consisting of the following steps: local invasion at the primary tumor site, intravasation and survival into the circulation, extravasation and survival at distant sites, adaptation to a foreign microenvironment and outgrowth of a metastasis. During every step of the metastatic cascade, cancer cells encounter normal host cells, such as immune cells. Interactions between disseminated cancer cells and normal host cells largely dictate the success of metastasis formation.

***MMTV-Neu* mouse tumor model**: A GEMM for $HER2^+$ breast cancer in which wild-type rat ERBB2 expression is driven by the Mouse Mammary Tumor Virus (MMTV) promoter, which is only active in the mammary gland. These mice develop multifocal tumors in all 10 mammary glands, as well as spontaneous lung metastases in most mice. They are maintained on the FVB/n background.

***MMTV-NeuT* mouse tumor model**: Similar to *MMTV-Neu* mice, this GEMM represents another model for $HER2^+$ breast cancer. However, a mutated form of the rat proto-oncogene, ERBB2, is expressed under control of the Mouse Mammary Tumor Virus (MMTV) promoter in this case. Multifocal tumors also arise in these mice from all five pairs of mammary glands and they develop spontaneous lung metastases. These mice are usually maintained on the BALB/c background.

***MMTV-PyMT* mouse tumor model:** A GEMM for mammary tumorigenesis. These mice transgenically express the viral oncogene Polyoma Middle T antigen under control of the

Mouse Mammary Tumor Virus (MMTV) promoter. These mice develop multifocal tumors in all 10 mammary glands, as well as spontaneous lung metastases.

Patient-derived xenograft (PDX) tumor models: Fresh tumor tissue from patients undergoing surgery is implanted into immunodeficient mice (usually NOD/SCID/*Il2rg*, otherwise known as NSG, mice) directly or following enzymatic digestion. Tumors can be grafted subcutaneously or orthotopically. PDX tumors are serially passaged in additional mice.

***Probasin-Cre4;Pten*$^{F/F}$ mouse tumor model:** A conditional GEMM for *Pten*-deficient prostate cancer, where loss of *Pten* expression is driven by the *Probasin* promoter. These mice develop prostatic intraepithelial neoplasia (PIN) lesions that progress to invasive adenocarcinomas.

Platinum compounds: A class of platinum-containing chemotherapy drugs that binds and crosslinks DNA, resulting in apoptosis. Examples of platinum compounds are cisplatin, carboplatin and oxaliplatin.

***RIP1-Tag5* mouse tumor model:** A conditional GEMM of pancreatic cancer, in which the Rat Insulin Promoter drives sporadic expression of SV40 large T antigen in a subset of pancreatic beta cells. Unlike *RIP-Tag2* mice that are systemically tolerant to SV40 large T antigen, these mice develop an autoimmune response against the oncogene-expressing beta cells.

Taxanes: A class of chemotherapy drugs that disrupts microtubule function, and thus inhibits mitosis. Taxanes were first derived from plants of the yew tree. Examples of taxanes are paclitaxel and docetaxel.

Tumor microenvironment: Besides cancer cells, many ‘normal’ cells are recruited to and activated in tumors. The tumor microenvironment is composed of many different types of immune cells, fibroblasts (referred to as cancer-associated fibroblasts), endothelial cells and other cells that normally reside in the organ afflicted by the tumor (e.g. adipocytes in breast cancer), soluble mediators and extracellular matrix (ECM). Throughout cancer progression, there is extensive crosstalk between normal cells, soluble mediators and cancer cells. These interactions largely dictate tumor behavior and therapy response. Each tumor type and each tumor stage is characterized by a unique tumor microenvironment.

Figure Legends

Figure 1. Categories of anti-cancer therapies and their targets. One of the first anti-cancer therapies, chemotherapy, was designed to target highly proliferating cancer cells, but over the last few decades, the arsenal of anti-cancer weapons has increased and now also includes stromal cell targets within the tumor microenvironment. Currently, cancer cells and stromal cells are targeted with chemotherapy, radiotherapy, targeted therapy – specific for oncogenes or hyperactive signaling pathways – vascular-targeting agents, T cell checkpoint inhibitors and immunomodulatory agents, among others. Examples are given of each anti-cancer therapy category based on their mention in the text, and the list is not inclusive of every anti-cancer therapy being tested in pre-clinical or clinical trials. As tumors are a collection of cancer and stromal cells, targeted cells responding to any given anti-cancer therapy through secretion of molecules or death may also affect their cellular neighbors within the tumor microenvironment indirectly.

Figure 2. The effects of anti-cancer therapies on immune cells. Anti-cancer therapies, such as chemotherapy, radiotherapy, targeted therapy and vascular-targeting agents, have been shown to modulate various immune cell populations in different ways. These include both indirect and direct effects and a few examples are illustrated. (*The importance of immunogenic cell death processes and $CD8^+$ T cell function in response to oxiliplatin and anthracyclines is largely based on transplantable tumor models [59, 60, 81], whereas response to oxiliplatin and anthracyclines in some GEMMs is not dependent on immunogenic cell death processes or $CD8^+$ T cell function [22, 23, 85].

Figure 3. Immune cell participation in metastasis at the primary tumor site and distant organs: potential therapeutic targets. Metastasis occurs through a cascade of events in which cancer cells escape from the primary tumor site, travel through the blood or lymph system, seed in distant organs or lymph nodes and grow out. Based on this cascade, experimental and clinical investigations are attempting to counteract metastasis by two major strategies that include preventing metastasis from occurring or combating established metastatic lesions. Pre-clinical evidence indicates that immune cells are important mediators of the metastatic cascade [17, 197], providing additional opportunities to incorporate immunotherapy and/or immunomodulatory agents into conventional treatment regimens. This figure highlights the studies we reference in the text regarding the role of immune cells in metastasis formation and the specific anti-cancer therapies used to block or reduce metastasis in mouse models. These studies show that immune cells promote or prevent metastasis at the primary tumor site or in distant organs. In GEMM and transplantable models of breast cancer, paclitaxel in combination with the TAM targeted antibody, anti-

CSF1R, as well as radiotherapy and anti-CTLA4 inhibits the formation of spontaneous metastasis [22, 23, 175, 202]. Neutralization of Angiopoietin 2 (ANGPT2) also reduces metastasis in breast cancer models by preventing TAM association with endothelial cells and angiogenesis [140] as well as CCL2-dependent recruitment of monocytes to metastatic lesions in lung [144, 145]. TAMs resident in pancreatic tumors suppress $CD8^+$ T cell functions, and targeting TAMs via anti-CSF1R together with gemcitabine decreases liver metastasis through reactivation of $CD8^+$ T cells [29]. Experiments carried out in melanoma metastasis models have shown that the BRAF inhibitor, PLX4720, and the cKIT inhibitor, imatinib, are effective against lung metastases via an NK cell-dependent mechanism [123, 128]. In addition, other mechanistic studies have identified several putative targets that may be effective at combating metastasis, including IL17-producing $\gamma\delta$ T cells [49] and pro-metastatic neutrophils [48, 49].

Table 1. Beneficial and antagonistic roles of immune cells in anti-cancer therapy response of various cancer mouse models. A checkmark indicates a confirmed role for an immune cell, whereas a dash represents a tested but unimportant role.

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